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(54) MICROORGANISMS OF CORYNEBACTERIUM WITH IMPROVED 5'-INOSINIC ACID PRODUCTIVITY, AND METHOD FOR PRODUCING NUCLEIC ACIDS USING SAME

(57) The present invention relates to a microorganisms of the genus Corynebacterium producing 5'-inosinic acid, in which the expression of genes encoding purine biosynthesis related enzymes is increased higher than the intrinsic expression, and to a method for producing 5'-inosinic acid, comprising culturing the microorganisms of the genus Corynebacterium with improved 5'-inosinic acid productivity.

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Description

BACKGROUND OF THE INVENTION

1. Field of the Invention

[0001] The present invention relates to a microorganism belonging to the genus *Corynebacterium* producing 5'-inosinic acid, in which the expression of genes encoding purine biosynthesis related enzymes is increased higher than the intrinsic expression, and a method for producing 5'-inosinic acid, comprising culturing the microorganism of the genus *Corynebacterium* with improved 5'-inosinic acid productivity.

2. Description of the Related Art

[0002] One of the nucleotide compounds, 5'-inosinic acid is an intermediate material of the metabolic system of nucleotide biosynthesis, which is used in a variety of fields such as foods, medicines, and other various medical areas and functions to play an important role in animal and plant physiology. In particular, 5'-inosinic acid is a nucleotide seasoning, which has drawn much attention as a savory seasoning, because it has synergistic effects when used with monosodium glutamate (MSG).

[0003] So far well known processes for producing 5'-inosinic acid include a process of enzymatically decomposing ribonucleic acid extracted from yeast cells (Japanese Published Examined Patent Application No. 1614/1957, etc), a process of chemically phosphorylating inosine produced by fermentation (Agric. Biol. Chem., 36, 1511(1972), etc) and a process of culturing a microorganism capable of producing 5'-inosinic acid and recovering inosine monophosphate (IMP) accumulated in the medium. Currently, the processes of producing 5'-inosinic acid using microorganisms are mostly used. The strains of the genus *Corynebacterium* are widely used as a microorganism for the production of 5'-inosinic acid, and for example, a method for producing 5'-inosinic acid by culturing *Corynebacterium ammoniagenes* is disclosed (Korean Patent Publication No. 2003-0042972).

[0004] To improve a production yield of 5'-inosinic acid by a microorganism, studies have been made to develop strains by increasing or decreasing activity or expression of the enzymes involved in the biosynthetic or degradative pathway of 5'-inosinic acid. Korean Patent No. 785248 discloses a microorganism in which a *purC* gene encoding phosphoribosylaminoimidazole succinocarboxamide synthetase is overexpressed in the purine biosynthetic pathway and a method for producing 5'-inosinic acid using the same. In addition, Korean Patent No. 857379 discloses a *Corynebacterium ammoniagenes* strain in which the *purKE* - encoded phosphoribosylaminoimidazole carboxylase is overexpressed and a method for producing high concentration of IMP in a high yield using the same.

[0005] However, there is still a need to develop a strain capable of producing 5'-inosinic acid in a higher yield and a method for producing 5'-inosinic acid using the same.

[0006] Therefore, the present inventors have conducted studies to develop a strain capable of producing 5'-inosinic acid with high productivity. As a result, they found that the productivity of 5'-inosinic acid can be improved by simultaneously increasing activities of the major enzymes involved in the purine biosynthesis pathway higher than the intrinsic activity, thereby completing the present invention.

SUMMARY OF THE INVENTION

[0007] An object of the present invention is to provide a microorganism of the genus *Corynebacterium* having improved 5'-inosinic acid productivity.

[0008] Another object of the present invention is to provide a method for producing 5'-inosinic acid using the microorganism of the genus *Corynebacterium* having improved 5'-inosinic acid productivity.

BRIEF DESCRIPTION OF THE DRAWINGS

[0009]

FIG. 1 shows a pDZ vector for chromosomal insertion into the microorganism of the genus *Corynebacterium*;
FIG. 2 shows a pDZ-2purFM vector for chromosomal insertion into the microorganism of the genus *Corynebacterium;*
FIG. 3 shows a pDZ-2purNH vector for chromosomal insertion into the microorganism of the genus *Corynebacterium;*
FIG. 4 shows a pDZ-2purSL vector for chromosomal insertion into the microorganism of the genus *Corynebacterium;*
FIG. 5 shows a pDZ-2purKE vector for chromosomal insertion into the microorganism of the genus *Corynebacterium;*
FIG. 6 shows a pDZ-2purC vector for chromosomal insertion into the microorganism of the genus *Corynebacterium*; and

FIG. 7 shows a pDZ-2prs vector for chromosomal insertion into the microorganism of the genus *Corynebacterium.*

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

[0010] In order to achieve the above objects, the present invention provides a microorganism belonging to the genus *Corynebacterium* producing 5'-inosinic acid, in which the expression of genes encoding purine biosynthesis related enzymes is increased higher than the intrinsic expression.

[0011] The microorganism of the genus *Corynebacterium* of the present invention has more improved 5'-inosinic acid productivity than a parental strain, because the expression of genes encoding purine biosynthesis related enzymes is increased higher than the intrinsic expression.

[0012] As used herein, the term "purine biosynthesis related enzyme" means an enzyme that catalyzes the reaction involved in the purine biosynthesis pathway producing a purine base as a final product, and includes phosphoribosylpyrophosphate amidotransferase, phosphoribosylglycinamide formyltransferase, phosphoribosylformylglycinamidin synthetase, phosphoribosylformylglycinamidin synthetase II, phosphoribosylaminoimidazole synthetase, phosphoribosylaminoimidazole carboxylase, phosphoribosyl aminoimidazole succinocarboxamide synthetase, inosinic acid cyclohydrolase, ribosephosphate pyrophosphokinase or the like.

[0013] In a specific embodiment of the present invention, the purine biosynthesis related enzymes may be a combination of one or more enzymes selected from the group consisting of phosphoribosylpyrophosphate amidotransferase, phosphoribosylglycinamide formyltransferase, phosphoribosylformylglycinamidin synthetase, phosphoribosylformylglycinamidin synthetase II, phosphoribosylaminoimidazole synthetase, phosphoribosylaminoimidazole carboxylase, phosphoribosyl aminoimidazole succinocarboxamide synthetase and inosinic acid cyclohydrolase, and ribosephosphate pyrophosphokinase.

[0014] In a specific embodiment of the present invention, the gene encoding purine biosynthesis related enzymes, of which expression is increased higher than the intrinsic expression, are a combination of a purN gene of SEQ ID NO. 36, which codes for phosphoribosylglycinamide formyltransferase, a purS gene of SEQ ID NO. 37, which codes for phosphoribosylformylglycinamidin synthetase, a purL gene of SEQ ID NO. 38, which codes for phosphoribosylformylglycinamidin synthetase II, a purKE gene of SEQ ID NO. 40, which codes for phosphoribosylaminoimidazole carboxylase, a purC of SEQ ID NO. 41, which codes for phosphoribosyl aminoimidazole succinocarboxamide synthetase, a purH gene of SEQ ID NO. 42, which codes for inosinic acid cyclohydrolase, and a prs gene of SEQ ID NO. 43, which codes for ribosephosphate pyrophosphokinase.

[0015] In a specific embodiment of the present invention, the gene encoding purine biosynthesis related enzymes, of which expression is increased higher than the intrinsic expression, are a combination of a purF gene of SEQ ID NO. 35, which codes for phosphoribosylpyrophosphate amidotransferase, a purN gene of SEQ ID NO. 36, which codes for phosphoribosylglycinamide formyltransferase, a purS gene of SEQ ID NO. 37, which codes for phosphoribosylformylglycinamidin synthetase, a purL gene of SEQ ID NO. 38, which codes for phosphoribosylformylglycinamidin synthetase II, a purM gene of SEQ ID NO. 39, which codes for phosphoribosylaminoimidazole synthetase, a purKE gene of SEQ ID NO. 40, which codes for phosphoribosylaminoimidazole carboxylase, a purC of SEQ ID NO. 41, which codes for phosphoribosyl aminoimidazole succinocarboxamide synthetase, a purH gene of SEQ ID NO. 42, which codes for inosinic acid cyclohydrolase, and a prs gene of SEQ ID NO. 43, which codes for ribosephosphate pyrophosphokinase.

[0016] As used herein, the term "increased higher than the intrinsic expression" means that the gene expression level is higher than that in naturally expressed in a microorganism or higher than that expressed in a parental strain, and includes an increase in the number (copy number) of the genes encoding corresponding enzyme and the expression level increased thereby or an increase in the expression level by mutation or an increase in the expression level by both of them.

[0017] In a specific embodiment of the present invention, the increase in the expression level of the gene encoding purine biosynthesis related enzyme includes the increase in the copy number of the gene by additionally introducing the corresponding foreign gene into a strain or by amplifying the intrinsic gene, or the increase in transcription efficiency or translation efficiency by mutation in the transcription or translation regulatory sequence, but is not limited thereto. The amplification of the intrinsic gene may be easily performed by a method known in the art, for example, by cultivation under a suitable selection pressure.

[0018] In a specific embodiment of the present invention, the expression level of the gene encoding purine biosynthesis related enzyme may be increased by additionally introducing the gene encoding purine biosynthesis related enzyme into a cell or by amplifying the intrinsic gene encoding the purine biosynthesis related enzyme.

[0019] In a specific embodiment of the present invention, the gene encoding purine biosynthesis related enzyme, of which the expression level is increased higher than the intrinsic expression, may exist as two or more copies in the microorganism of the genus *Corynebacterium* having improved 5'-inosinic acid productivity by introducing one or more copies into a cell, in addition to the corresponding intrinsic gene.

[0020] In a specific embodiment of the present invention, the gene encoding purine biosynthesis related enzyme is introduced into the microorganism of the genus *Corynebacterium* having improved 5'-inosinic acid productivity by transformation using a recombinant vector containing two copies of the corresponding gene that are consecutively arranged.

[0021] In a specific embodiment of the present invention, the recombinant vector used for preparation of the microorganism of the genus *Corynebacterium* having improved 5'-inosinic acid productivity may be selected from the group consisting of pDZ-2purFM, pDZ-2purNH, pDZ-2purSL, pDZ-2purKE, pDZ-2purC, and pDZ-2prs recombinant vectors, which have the cleavage maps of FIGs. 2 to 7 respectively, depending on the genes introduced.

[0022] In a specific embodiment of the present invention, the microorganism of the genus *Corynebacterium* having improved 5'-inosinic acid productivity may be derived from *Corynebacterium* microorganisms capable of producing 5'-inosinic acid. For example, the microorganism of the genus *Corynebacterium* having improved 5'-inosinic acid productivity according to the present invention may be derived from *Corynebacterium ammoniagenes* ATCC6872, *Corynebacterium thermoaminogenes* FERM BP-1539, *Corynebacterium glutamicum* ATCC13032, *Brevibacterium flavum* ATCC14067, *Brevibacterium lactofermentum* ATCC13869, and strains prepared therefrom.

[0023] In a specific embodiment of the present invention, the microorganism of the genus *Corynebacterium* having improved 5'-inosinic acid productivity may include two or more copies of the gene encoding purine biosynthesis related enzyme.

[0024] In a specific embodiment of the present invention, the microorganism of the genus *Corynebacterium* having improved 5'-inosinic acid productivity may be *Corynebacterium ammoniagenes,* and more preferably a transformed *Corynebacterium ammoniagenes,* in which the activity of a combination of the prs gene and one or more genes selected from the group consisting of purF, purN, purS, purL, purM, purKE, purC, and purH is increased to produce high concentration of 5'-inosinic acid.

[0025] In a specific embodiment of the present invention, the microorganism of the genus *Corynebacterium* having improved 5'-inosinic acid productivity may be a strain, in which the 5'-inosinic acid-producing *Corynebacterium ammoniagenes* CJIP2401 (KCCM-10610) strain is introduced with each of the pDZ-2purFM, pDZ-2purNH, pDZ-2purSL, pDZ-2purKE, pDZ-2purC, and pDZ-2prs recombinant vectors having the cleavage maps of FIGs. 2, 3, 4, 5, 6, and 7 in order or in combination, and one of two copies of the introduced purF, purN, purS, purL, purM, purKE, purC, purH and prs genes are substituted for the corresponding intrinsic genes by homologous recombination, and thus each two copies of the purF, purN, purS, purL, purM, purKE, purC, purH, and prs genes are inserted into the strain.

[0026] In a specific embodiment of the present invention, the microorganism of the genus *Corynebacterium* having improved 5'-inosinic acid productivity may be *Corynebacterium ammoniagenes* containing two copies of the genes encoding purine biosynthesis related enzymes that are a combination of the purN gene of SEQ ID NO. 36, which codes for phosphoribosylglycinamide formyltransferase, the purS gene of SEQ ID NO. 37, which codes for phosphoribosylformylglycinamidin synthetase, the purL gene of SEQ ID NO. 38, which codes for phosphoribosylformylglycinamidin synthetase II, the purKE gene of SEQ ID NO. 40, which codes for phosphoribosylaminoimidazole carboxylase, the purC of SEQ ID NO. 41, which codes for phosphoribosyl aminoimidazole succinocarboxamide synthetase, the purH gene of SEQ ID NO. 42, which codes for inosinic acid cyclohydrolase, and the prs gene of SEQ ID NO. 43, which codes for ribosephosphate pyrophosphokinase, and preferably *Corynebacterium ammoniagenes* CN01-0120.

[0027] In a specific embodiment of the present invention, the microorganism of the genus *Corynebacterium* having improved 5'-inosinic acid productivity may be *Corynebacterium ammoniagenes* containing two copies of the genes encoding purine biosynthesis related enzymes that are a combination of the purF of SEQ ID NO. 35, which codes for phosphoribosylpyrophosphate amidotransferase, the purN gene of SEQ ID NO. 36, which codes for phosphoribosylglycinamide formyltransferase, the purS gene of SEQ ID NO. 37, which codes for phosphoribosylformylglycinamidin synthetase, the purL gene of SEQ ID NO. 38, which codes for phosphoribosylformylglycinamidin synthetase II, the purM of SEQ ID NO. 39, which codes for phosphoribosylaminoimidazole synthetase, the purKE gene of SEQ ID NO. 40, which codes for phosphoribosylaminoimidazole carboxylase, the purC of SEQ ID NO. 41, which codes for phosphoribosyl aminoimidazole succinocarboxamide synthetase, the purH gene of SEQ ID NO. 42, which codes for inosinic acid cyclohydrolase, and the prs gene of SEQ ID NO. 43, which codes for ribosephosphate pyrophosphokinase, and preferably *Corynebacterium ammoniagenes* CN01-0316 (KCCM 10992P).

[0028] Further, the present invention provides a method for producing 5'-inosinic acid, comprising the steps of culturing the microorganism belonging to the genus *Corynebacterium* producing 5'-inosinic acid, in which the expression of a gene encoding purine biosynthesis related enzyme is increased higher than the intrinsic expression, and recovering 5'-inosinic acid from the culture medium.

[0029] In the method for producing 5'-inosinic acid of the present invention, the medium and other culture conditions used for the cultivation of the microorganism of the genus *Corynebacterium* may be the same as those typically used in the cultivation of the microorganism of the genus *Corynebacterium*, and easily selected and adjusted by those skilled in the art. In addition, the cultivation may be performed by any cultivation method known to those skilled in the art, for example, batch, continuous, and fed-batch culture, but is not limited thereto.

[0030] In a specific embodiment of the present invention, the microorganism belonging to the genus *Corynebacterium* producing 5'-inosinic acid may be *Corynebacterium ammoniagenes*.

[0031] In a specific embodiment of the present invention, the microorganism belonging to the genus *Corynebacterium* producing 5'-inosinic acid may be *Corynebacterium ammoniagenes* CN01-0120 or *Corynebacterium ammoniagenes* CN01-0316 (KCCM 10992P).

[0032] In a specific embodiment of the present invention, culturing the microorganism of the genus *Corynebacterium* is performed by culturing the strain in a conventional medium containing suitable carbon sources, nitrogen sources, amino acids, vitamins or the like under aerobic conditions by adjusting temperature, pH or the like.

[0033] As a carbon source, carbohydrates such as glucose and fructose may be used. As a nitrogen source, various inorganic nitrogen sources such as ammonia, ammonium chloride, and ammonium sulphate may be used, and organic nitrogen sources such as peptone, NZ-amine, beef extract, yeast extract, corn steep liquor, casein hydrolysate, fish or fish meal, and defatted soybean cake or meal may be used. Examples of the inorganic compounds include potassium monohydrogen phosphate, potassium dihydrogen phosphate, magnesium sulfate, ferrous sulfate, manganese sulfate, and calcium carbonate. When needed, vitamins and auxotrophic bases may be used.

[0034] The cultivation is performed under aerobic conditions, for example, by shaking culture or stirring culture, preferably at a temperature of 28 to 36°C. During the cultivation, the pH is preferably maintained within the range of pH 6 to 8. The cultivation may be performed for 4 to 6 days.

[0035] Hereinafter, the present invention will be described in more detail with reference to Examples. However, these Examples are for illustrative purposes only, and the invention is not intended to be limited by these Examples.

Example 1. Insertion of genes encoding purine biosynthesis related enzymes using vector (pDZ) for chromosomal insertion and Development of strain producing high yield of 5'-inosinic acid thereby

[0036] In order to insert a foreign gene into the chromosome of *Corynebacterium ammoniagenes* strain, a pDZ-based recombinant vector containing two consecutive copies of the corresponding gene was used. The pDZ vector is a vector for chromosomal insertion into the microorganism of the genus *Corynebacterium,* and was prepared by the method disclosed in Korean Patent Publication No. 2008-0025355 incorporated by reference herein. FIG. 1 is a schematic diagram showing the structure of the pDZ vector.

[0037] In the following (1) to (6), recombinant vectors were prepared, in which the recombinant vectors function to insert the gene encoding purine biosynthesis related enzyme into the chromosome of the microorganism of the genus *Corynebacterium* to obtain two copies of each gene. Transformation by each recombinant vector and selection of transformants were performed as follows.

[0038] The 5'-inosinic acid-producing strain, *Corynebacterium ammoniagenes* CJIP2401 (KCCM-10610) was transformed with the pDZ recombinant vector containing the desired gene encoding purine biosynthesis related enzyme by electroporation, and then strains, in which the gene carried by the vector is inserted into their chromosome by homologous recombination , were selected on a selection medium containing 25 mg/l of kanamycin. The successful chromosomal insertion of the vector was confirmed by the color of the colonies on a solid medium (1% beef extract, 1% yeast extract, 1% peptone, 0.25% sodium chloride, 1% adenine, 1% guanine, 1.5% agarose) containing X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside). That is, blue colonies were selected as a transformant, in which the vector was inserted into the chromosome. The strain, in which the vector was inserted into its chromosome via a first crossover, was shaking-cultured (30°C, 8 hours) in a nutrient medium (1% glucose, 1% beef extract, 1% yeast extract, 1% peptone, 0.25% sodium chloride, 1% adenine, 1% guanine). Then, the cultured strain was serially diluted from 10^{-4} to 10^{-10}, and the diluted culture was plated on a solid medium containing X-gal. Most colonies exhibited blue color, but white colonies also existed at a low level. By selecting the white colonies, strains in which the sequence of the vector was removed from the chromosome via a second crossover were selected. The selected strain was identified as a final strain by a susceptibility test for kanamycin and a gene sequence analysis by PCR.

(1) Cloning of purFM gene and Construction of recombinant vector (pDZ-2purFM)

[0039] The purF and purM genes are located close to each other on the chromosome of the microorganism of the genus *Corynebacterium,* and thus a purFM vector containing both of the genes and the promoter region was constructed in order to express both of the genes at the same time.

[0040] The chromosome was isolated from *Corynebacterium ammoniagenes* CJIP2401 producing 5'-inosinic acid, and Polymerase Chain Reaction (PCR) was performed using the chromosome as a template in order to obtain purFM, namely, a fragment containing the consecutively arranged purF and purM. *PfuUltra*™ High-Fidelity DNA Polymerase (Stratagene) was used as a polymerase, and Polymerase Chain Reaction was performed with 30 cycles of denaturing at 96°C for 30 sec, annealing at 53°C for 30 sec, and polymerization at 72°C for 2 min. As a result, two purFM genes containing the promoter region (purFM-A, purFM-B) were obtained. The purFM-A was amplified using the primers of SEQ ID NOs. 1 and 2, and the purFM-B was amplified using the primers of SEQ ID NOs. 3 and 4. The amplification products were cloned into an *E.coli* vector pCR2.1 using a TOPO Cloning Kit (Invitrogen) so as to obtain pCR-purFM-

A and pCR-purFM-B vectors, respectively. The pCR vectors were treated with restriction enzymes contained in each end of the purFM-A and purFM-B (purFM-A: EcoRI+XbaI, purFM-B: XbaI+HindIII), and each purFM gene was separated from the pCR vectors. Thereafter, the pDZ vector treated with restriction enzymes, *Eco*RI and *Hind*III was cloned by 3-piece ligation so as to construct a pDZ-2purFM recombinant vector where two purFM genes are consecutively cloned. FIG. 2 shows a pDZ-2purFM vector for chromosomal insertion into *Corynebacterium.*

[0041] The 5'-inosinic acid-producing strain, *Corynebacterium ammoniagenes* CJIP2401 was transformed with the pDZ-2purFM vector by electroporation, and one purFM gene is additionally inserted next to the intrinsic purFM gene on the chromosome via a second crossover, so as to obtain a strain having total two copies. The consecutively inserted purFM genes were identified by PCR using the primers of SEQ ID NOs. 5 and 6 which are able to amplify the regions of connecting two purFM genes.

(2) Cloning of purNH gene and Construction of recombinant vector (pDZ-2purNH), preparation of purNH-inserted strain

[0042] The purN and purH genes are located close to each other on the chromosome of the microorganism of the genus *Corynebacterium,* and thus a purNH vector containing the promoter region was constructed in order to express both of the genes at the same time.

[0043] The chromosome was isolated from *Corynebacterium ammoniagenes* CJIP2401 producing 5'-inosinic acid, and Polymerase Chain Reaction (PCR) was performed using the chromosome as a template in order to obtain purNH, namely, a fragment containing the consecutively arranged purN and purH. *PfuUltra*™ High-Fidelity DNA Polymerase (Stratagene) was used as a polymerase, and Polymerase Chain Reaction was performed with 30 cycles of denaturing at 96°C for 30 sec, annealing at 53°C for 30 sec, and polymerization at 72°C for 2 min. As a result, two purNH genes containing the promoter region (purNH-A, purNH-B) were obtained. The purNH-A was amplified using the primers of SEQ ID NOs. 7 and 8, and the purNH-B was amplified using the primers of SEQ ID NOs. 8 and 9. The amplification products were cloned into an *E.coli* vector pCR2.1 using a TOPO Cloning Kit (Invitrogen) so as to obtain pCR-purNH-A and pCR-purNH-B vectors, respectively. The pCR vectors were treated with restriction enzymes contained in each end of the purNH-A and purNH-B (purNH-A: BamHI+SalI, purNH-B: SalI), and each purNH gene was separated from the pCR vectors. Thereafter, the pDZ vector treated with restriction enzymes, *BamH*I and *Sal*I was cloned by 3-piece ligation so as to construct a pDZ-2purNH recombinant vector where two purNH genes are consecutively cloned. FIG. 3 shows a pDZ-2purNH vector for chromosomal insertion into *Corynebacterium.*

[0044] The 5'-inosinic acid-producing strain, *Corynebacterium ammoniagenes* CJIP2401 was transformed with the pDZ-2purNH vector by electroporation, and one purNH gene is additionally inserted next to the intrinsic purNH gene on the chromosome via a second crossover, so as to obtain a strain having total two copies. The consecutively inserted purNH genes were identified by PCR using the primers of SEQ ID NOs. 10 and 11 which are able to amplify the regions of connecting two purNH genes.

(3) Cloning of purSL gene and Construction of recombinant vector (pDZ-2purSL), preparation of purSL-inserted strain

[0045] The purS and purL genes are located close to each other on the chromosome of the microorganism of the genus *Corynebacterium*, and thus a purSL vector containing the promoter region was constructed in order to express both of the genes at the same time.

[0046] The chromosome was isolated from *Corynebacterium ammoniagenes* CJIP2401 producing 5'-inosinic acid, and Polymerase Chain Reaction (PCR) was performed using the chromosome as a template in order to obtain purSL, namely, a fragment containing the consecutively arranged purS and purL. *PfuUltra*™ High-Fidelity DNA Polymerase (Stratagene) was used as a polymerase, and Polymerase Chain Reaction was performed with 30 cycles of denaturing at 96°C for 30 sec, annealing at 53°C for 30 sec, and polymerization at 72°C for 2 min. As a result, two purSL genes containing the promoter region (purSL-A, purSL-B) were obtained. The purSL-A was amplified using the primers of SEQ ID NOs. 12 and 13, and the purSL-B was amplified using the primers of SEQ ID NOs. 14 and 15. The amplification products were cloned into an *E.coli* vector pCR2.1 using a TOPO Cloning Kit (Invitrogen) so as to obtain pCR-purSL-A and pCR-purSL-B vectors, respectively. The pCR vectors were treated with restriction enzymes contained in each end of the purSL-A and purSL-B (purSL-A: BamHI+SalI, purSL-B: SalI+BamHI), and each purSL gene was separated from the pCR vectors. Thereafter, the pDZ vector treated with restriction enzyme, BamHI was cloned by 3-piece ligation so as to construct a pDZ-2purSL recombinant vector where two purSL genes are consecutively cloned. FIG. 4 shows a pDZ-2purSL vector for chromosomal insertion into *Corynebacterium.*

[0047] The 5'-inosinic acid-producing strain, *Corynebacterium ammoniagenes* CJIP2401 was transformed with the pDZ-2purSL vector by electroporation, and one purSL gene is additionally inserted next to the intrinsic purSL gene on the chromosome via a second crossover, so as to obtain a strain having total two copies. The consecutively inserted purSL genes were identified by PCR using the primers of SEQ ID NOs. 16 and 17 which are able to amplify the regions of connecting two purSL genes.

(4) Cloning of purKE gene and Construction of recombinant vector (pDZ-2purKE), preparation of purKE-inserted strain

[0048] The purK and purE genes are located close to each other on the chromosome of the microorganism of the genus *Corynebacterium,* and thus a purKE vector containing the promoter region was constructed in order to express both of the genes at the same time.

[0049] The chromosome was isolated from *Corynebacterium ammoniagenes* CJIP2401 producing 5'-inosinic acid, and Polymerase Chain Reaction (PCR) was performed using the chromosome as a template in order to obtain purKE, namely, a fragment containing the consecutively arranged purK and purE. *PfuUltra*™ High-Fidelity DNA Polymerase (Stratagene) was used as a polymerase, and Polymerase Chain Reaction was performed with 30 cycles of denaturing at 96°C for 30 sec, annealing at 53°C for 30 sec, and polymerization at 72°C for 2 min. As a result, two purKE genes containing the promoter region (purKE-A, purKE-B) were obtained. The purKE-A was amplified using the primers of SEQ ID NOs. 18 and 19, and the purKE-B was amplified using the primers of SEQ ID NOs. 20 and 21. The amplification products were cloned into an *E.coli* vector pCR2.1 using a TOPO Cloning Kit (Invitrogen) so as to obtain pCR-purKE-A and pCR-purKE-B vectors, respectively. The pCR vectors were treated with restriction enzymes contained in each end of the purKE-A and purKE-B (purKE-A: BamHI+KpnI, purKE-B: KpnI+XbaI), and each purKE gene was separated from the pCR vectors. Thereafter, the pDZ vector treated with restriction enzymes, BamHI and XbaI was cloned by 3-piece ligation so as to construct a pDZ-2purKE recombinant vector where two purKE genes are consecutively cloned. FIG. 5 shows a pDZ-2purKE vector for chromosomal insertion into *Corynebacterium.*

[0050] The 5'-inosinic acid-producing strain, *Corynebacterium ammoniagenes* CJIP2401 was transformed with the pDZ-2purKE vector by electroporation, and one purKE gene is additionally inserted next to the intrinsic purKE gene on the chromosome via a second crossover, so as to obtain a strain having total two copies. The consecutively inserted purKE genes were identified by PCR using the primers of SEQ ID NOs. 22 and 23 which are able to amplify the regions of connecting two purKE genes.

(5) Cloning of purC gene and Construction of recombinant vector (pDZ-2purC), preparation of purC-inserted strain

[0051] The chromosome was isolated from *Corynebacterium ammoniagenes* CJIP2401, and Polymerase Chain Reaction (PCR) was performed using the chromosome as a template in order to obtain purC. *PfuUltra*™ High-Fidelity DNA Polymerase was used as a polymerase, and Polymerase Chain Reaction was performed with 30 cycles of denaturing at 96°C for 30 sec, annealing at 53°C for 30 sec, and polymerization at 72°C for 2 min. As a result, two purC genes containing the promoter region (purC-A, purC-B) were obtained. The purC-A was amplified using the primers of SEQ ID NOs. 24 and 25, and the purC-B was amplified using the primers of SEQ ID NOs. 25 and 26. The amplification products were cloned into an *E.coli* vector pCR2.1 using a TOPO Cloning Kit so as to obtain pCR-purC-A and pCR-purC-B vectors, respectively. The pCR vectors were treated with restriction enzymes contained in each end of the purC-A and purC-B (purC-A: BamHI+SalI, purC-B: SalI), and each purC gene was separated from the pCR vectors. Thereafter, the pDZ vector treated with restriction enzymes, BamHI and SalI was cloned by 3-piece ligation so as to construct a pDZ-2purC recombinant vector where two purC genes are consecutively cloned. FIG. 6 shows a pDZ-2purC vector for chromosomal insertion into *Corynebacterium.*

[0052] The 5'-inosinic acid-producing strain, *Corynebacterium ammoniagenes* CJIP2401 was transformed with the pDZ-2purC vector by electroporation, and one purC gene is additionally inserted next to the intrinsic purC gene on the chromosome via a second crossover, so as to obtain a strain having total two copies. The consecutively inserted purC genes were identified by PCR using the primers of SEQ ID NOs. 27 and 28 which are able to amplify the regions of connecting two purC genes.

(6) Cloning of prs gene and Construction of recombinant vector (pDZ-2prs), preparation of prs-inserted strain

[0053] The chromosome was isolated from *Corynebacterium ammoniagenes* CJIP2401, and Polymerase Chain Reaction (PCR) was performed using the chromosome as a template in order to obtain prs. *PfuUltra*™ High-Fidelity DNA Polymerase was used as a polymerase, and Polymerase Chain Reaction was performed with 30 cycles of denaturing at 96°C for 30 sec, annealing at 53°C for 30 sec, and polymerization at 72°C for 2 min. As a result, two prs genes containing the promoter region (prs-A, prs-B) were obtained. The prs-A was amplified using the primers of SEQ ID NOs. 29 and 30, and the prs-B was amplified using the primers of SEQ ID NOs. 31 and 32. The amplification products were cloned into an *E.coli* vector pCR2.1 using a TOPO Cloning Kit so as to obtain pCR-prs-A and pCR-prs-B vectors, respectively. The pCR vectors were treated with restriction enzymes contained in each end of the prs-A and prs-B (prs-A: BamHI+SpeI, prs-B: SpeI+PstI), and each prs gene was separated from the pCR vectors. Thereafter, the pDZ vector treated with restriction enzymes, BamHI and PstI was cloned by 3-piece ligation so as to construct a pDZ-2prs recombinant vector where two prs genes are consecutively cloned. FIG. 7 shows a pDZ-2prs vector for chromosomal insertion into *Corynebacterium.*

[0054] The 5'-inosinic acid-producing strain, *Corynebacterium ammoniagenes* CJIP2401 was transformed with the pDZ-2prs vector by electroporation, and one prs gene is additionally inserted next to the intrinsic prs gene on the chromosome via a second crossover, so as to obtain a strain having total two copies. The consecutively inserted prs genes were identified by PCR using the primers of SEQ ID NOs. 33 and 34 which are able to amplify the regions of connecting two prs genes.

(7) Development of strain producing high yield of 5'-inosinic acid by enhancement of purine biosynthesis

[0055] Combinations of pDZ-2purFM, pDZ-2purNH, pDZ-2purSL, pDZ-2purKE, pDZ-2purC, and pDZ-2prs vectors constructed in (1) to (6) were introduced into the 5'-inosinic acid-producing *Corynebacterium ammoniagenes* CJIP2401. The introduction order of the vectors was randomly selected, and introduction method and identification are the same as the above.

[0056] The *Corynebacterium ammoniagenes* CJIP2401 was used as a parental strain, and transformed with a combination of pDZ-2purNH, pDZ-2purSL, pDZ-2purKE, pDZ-2purC, and pDZ-2prs, and a combination of pDZ-2purNH, pDZ-2purSL, pDZ-2purKE, pDZ-2purC, pDZ-2purFM and pDZ-2prs to obtain *Corynebacterium ammoniagenes* CN01-0120 (2purNH + 2purSL + 2purKE + 2purC + 2prs) and *Corynebacterium ammoniagenes* CN01-0316 (2purNH + 2purSL + 2purKE + 2purC + 2purFM + 2prs), which contain two copies of the genes encoding the major enzymes involved in the purine biosynthetic pathway.

Example 2. Fermentation titer test of recombinant *Corynebacterium ammoniagenes*

[0057] Each 3 ml of the seed medium with the following composition was distributed into test tubes having a diameter of 18 mm, and sterilized under pressure. Then, the parental strain *Corynebacterium ammoniagenes* CJIP2401, and the *Corynebacterium ammoniagenes* CN01-0120 and *Corynebacterium ammoniagenes* CN01-0316 prepared in Example 1 were inoculated, and shaking-cultured at 30°C for 24 hours to be used as a seed culture. Each 27 ml of the fermentation medium with the following composition was distributed into 500 ml Erlenmeyer shake flasks and sterilized under pressure at 120°C for 10 minutes, and each 3 ml of the seed culture was inoculated thereto and shaking-cultured for 5 to 6 days. The cultivation was carried out under the conditions of 200 rpm, 32°C, and pH 7.2

[0058] The seed medium and the fermentation medium have the following compositions.

Seed medium: 1% glucose, 1% peptone, 1% beef extract, 1% yeast extract, 0.25% sodium chloride, 100 mg/l adenine, 100 mg/l guanine, pH7.2

Flask fermentation medium: 0.1% sodium glutamate, 1% ammonium chloride, 1.2% magnesium sulfate, 0.01% calcium chloride, 20 mg/l iron sulfate, 20 mg/l manganese sulfate, 20 mg/l zinc sulfate, 5 mg/l copper sulfate, 23 mg/l L-cysteine, 24 mg/l alanine, 8 mg/l nicotinic acid, 45 μg/l biotin, 5 mg/l thiamine hydrochloride, 30 mg/l adenine, 1.9% phosphoric acid (85%), 4.2% glucose, and 2.4% raw sugar

[0059] After completion of the cultivation, the productivity of 5'-inosinic acid was measured by HPLC, and the accumulation amount of 5'-inosinic acid in the culture medium is shown in the following Table.

[Table 1]

Strain name	Cell OD (5 days after culture)	Productivity (g/l/hr) (5 days after culture)
Control group (CJIP2401)	31.2	0.136
CN01-0120	31.8	0.155
CN01-0316	31.3	0.149

[0060] The accumulation amount of 5'-inosinic acid in the culture medium was compared with that of the parental strain, *Corynebacterium ammoniagenes* CJIP2401. As a result, in *Corynebacterium ammoniagenes* CN01-0120 and *Corynebacterium ammoniagenes* CN01-0316, their 5'-inosinic acid productivity per hour was found to be increased to 10.9 - 11.4% under the same conditions, compared to the parental strain, *Corynebacterium ammoniagenes* CJIP2401.

[0061] *Corynebacterium ammoniagenes* CN01-0316 having improved 5'-inosinic acid productivity by increasing the activity of purine biosynthesis related enzymes was deposited in the Korean Culture Center of Microorganisms (KCCM) located at Hongje 1-dong, Seodaemun-gu, Seoul, with the Accession No. KCCM 10992P on Feb. 19, 2009 under the Budapest treaty.

Effect of the invention

[0062] The microorganism belonging to the genus *Corynebacterium* producing 5'-inosinic acid according to the present invention, in which the expression of gene encoding purine biosynthesis related enzymes is increased higher than the intrinsic expression, can be used to produce 5'-inosinic acid in a high concentration and a high yield, thereby reducing production costs.

ITEMS

[0063]

Item 1. A microorganism belonging to the genus *Corynebacterium* producing 5'-inosinic acid, in which the expression level of genes encoding purine biosynthesis related enzymes is increased higher than the intrinsic expression level, wherein the purine biosynthesis related enzymes are a combination of ribosephosphate pyrophosphokinase and one or more enzymes selected from the group consisting of phosphoribosylpyrophosphate amidotransferase, phosphoribosylglycinamide formyltransferase, phosphoribosylformylglycinamidin synthetase, phosphoribosylformylglycinamidin synthetase II, phosphoribosylaminoimidazole synthetase, phosphoribosylaminoimidazole carboxylase, phosphoribosyl aminoimidazole succinocarboxamide synthetase, and inosinic acid cyclohydrolase.

Item 2. The microorganism belonging to the genus *Corynebacterium* according to Item 1, wherein the genes encoding purine biosynthesis related enzymes are a combination of a purN gene of SEQ ID NO. 36, which codes for phosphoribosylglycinamide formyltransferase, a purS gene of SEQ ID NO. 37, which codes for phosphoribosylformylglycinamidin synthetase, a purL gene of SEQ ID NO. 38, which codes for phosphoribosylformylglycinamidin synthetase II, a purKE gene of SEQ ID NO. 40, which codes for phosphoribosylaminoimidazole carboxylase, a purC of SEQ ID NO. 41, which codes for phosphoribosyl aminoimidazole succinocarboxamide synthetase, a purH gene of SEQ ID NO. 42, which codes for inosinic acid cyclohydrolase, and a prs gene of SEQ ID NO. 43, which codes for ribosephosphate pyrophosphokinase.

Item 3. The microorganism belonging to the genus *Corynebacterium* according to Item 1, wherein the genes encoding purine biosynthesis related enzymes are a combination of a purF gene of SEQ ID NO. 35, which codes for phosphoribosylpyrophosphate amidotransferase, a purN gene of SEQ ID NO. 36, which codes for phosphoribosylglycinamide formyltransferase, a purS gene of SEQ ID NO. 37, which codes for phosphoribosylformylglycinamidin synthetase, a purL gene of SEQ ID NO. 38, which codes for phosphoribosylformylglycinamidin synthetase II, a purM gene of SEQ ID NO. 39, which codes for phosphoribosylaminoimidazole synthetase, a purKE gene of SEQ ID NO. 40, which codes for phosphoribosylaminoimidazole carboxylase, a purC of SEQ ID NO. 41, which codes for phosphoribosyl aminoimidazole succinocarboxamide synthetase, a purH gene of SEQ ID NO. 42, which codes for inosinic acid cyclohydrolase, and a prs gene of SEQ ID NO. 43, which codes for ribosephosphate pyrophosphokinase.

Item 4. The microorganism belonging to the genus *Corynebacterium* according to Item 1, wherein the expression level of the gene encoding purine biosynthesis related enzyme is increased by additionally introducing the gene encoding purine biosynthesis related enzyme into a cell or by amplifying the intrinsic gene encoding purine biosynthesis related enzyme.

Item 5. The microorganism belonging to the genus *Coxynebacterium* according to Item 4, wherein the gene encoding purine biosynthesis related enzyme exists as two or more copies by introducing one or more copies into a cell, in addition to the corresponding intrinsic gene.

Item 6. The microorganism belonging to the genus *Corynebacterium* according to Item 5, wherein introduction of the gene encoding purine biosynthesis related enzyme into the cell is performed by transformation using a recombinant vector containing two copies of the corresponding gene that are consecutively arranged.

Item 7. The microorganism belonging to the genus *Corynebacterium* according to Item 6, wherein the recombinant vector is selected from the group consisting of pDZ-2purFM, pDZ-2purNH, pDZ-2purSL, pDZ-2purKE, pDZ-2purC, and pDZ-2prs that have the cleavage maps of FIGs. 2 to 7, respectively.

Item 8. The microorganism belonging to the genus *Corynebacterium* according to Item 1, wherein the microorganism of the genus *Corynebacterium* is *Corynebacterium ammoniagenes*.

Item 9. The microorganism belonging to the genus *Corynebacterium* according to Item 2, wherein the microorganism of the genus *Corynebacterium* is *Corynebacterium ammoniagenes* CN01-0120.

Item 10. The microorganism belonging to the genus *Corynebacterium* according to Item 3, wherein the microorganism of the genus *Corynebacterium* is *Corynebacterium ammoniagenes* CN01-0316 (KCCM 10992P).

Item 11. A method for producing 5'-inosinic acid, comprising culturing the microorganism belonging to the genus *Corynebacterium* according to any one of Items 1 to 10, and recovering 5'-inosinic acid from the culture medium.

<110> CJ CheilJedang Coroporation

<120> MICROORGANISMS OF CORYNEBACTERIUM WITH IMPROVED 5'-INOSINIC ACID PRODUCTIVITY, AND METHOD FOR PRODUCING NUCLEIC ACIDS USING SAME

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Claims

1. A microorganism belonging to the genus *Corynebacterium* producing 5'-inosinic acid, in which the expression level of genes encoding purine biosynthesis consisting of a prs gene encoding ribosephosphate pyrophosphokinase, a purN gene encoding phosphoribosylglycinamide formyltransferase, a purS gene encoding phosphoribosylformylglycinamidin synthetase, a purL gene encoding phosphoribosylformylglycinamidin synthetase II, a purKE gene encoding phosphoribosylaminoimidazole carboxylase, a purC gene encoding phosphoribosyl aminoimidazole succinocarboxamide synthetase, and a purH gene encoding inosinic acid cyclohydrolase is increased higher than the intrinsic expression level of said genes by an increase of the copy number of said genes or by an increase of the transcription or translation efficiency of said genes.

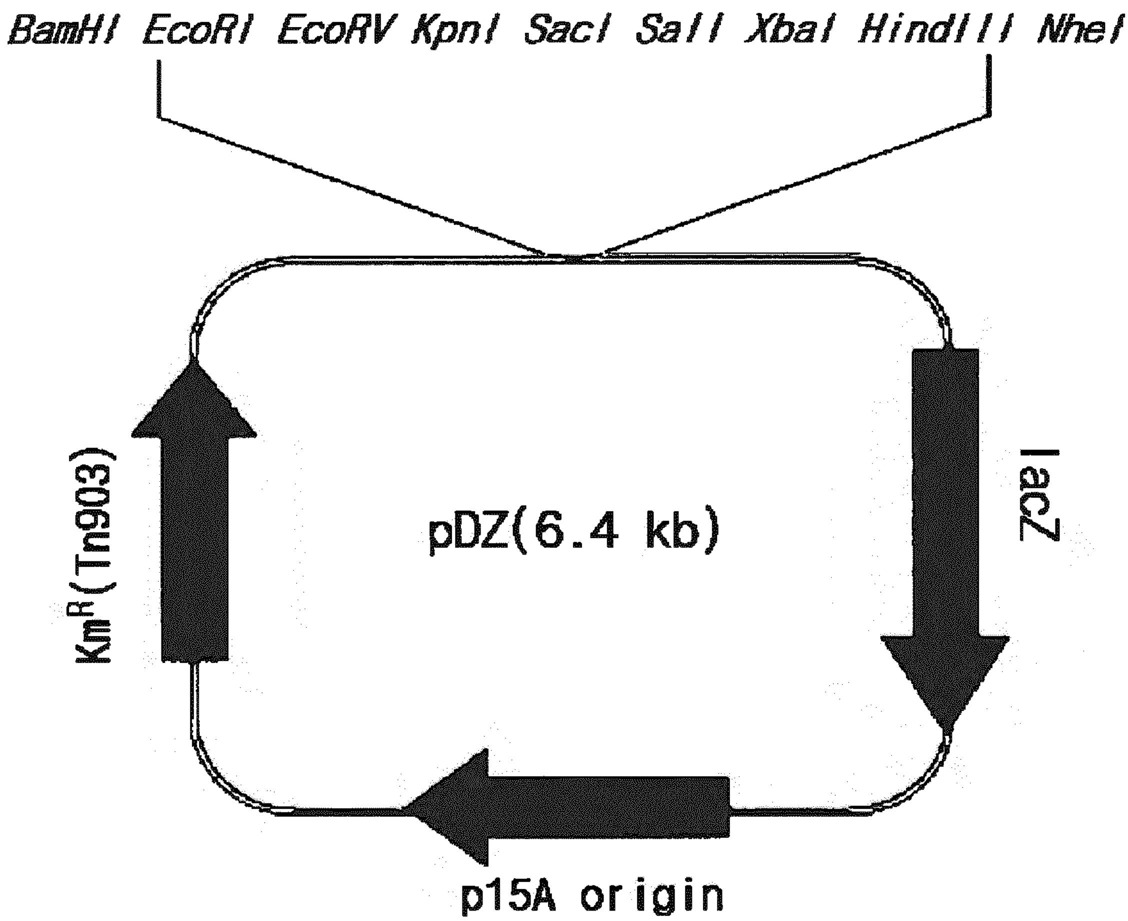
2. The microorganism according to claim 1, wherein the expression level of a purF gene encoding phosphoribosylpyrophosphate amidotransferase and a purM gene encoding phosphoribosylaminoimidazole synthetase is further increased higher than the intrinsic expression level of said genes by an increase of the copy number of said genes or by an increase of the transcription or translation efficiency of said genes.

3. The microorganism according to claim 1 or 2, wherein the purF gene is represented by SEQ ID NO. 35, the purN gene is represented by SEQ ID NO. 36, the purS gene is represented by SEQ ID NO. 37, the purL gene is represented by SEQ ID NO. 38, the purM gene is represented by SEQ ID NO. 39, the purKE gene is represented by SEQ ID NO. 40, the purC is represented by SEQ ID NO. 41, the purH gene is represented by SEQ ID NO. 42, and the prs gene is represented by SEQ ID NO. 43.

4. The microorganism according to any one of claims 1 to 3, wherein the microorganism is *Corynebacterium ammoniagenes*.

5. A method for producing 5'-inosinic acid, comprising culturing the microorganism of any one of claims 1 to 4 in a culture medium, and recovering 5'-inosinic acid from the culture medium.

[FIG. 1]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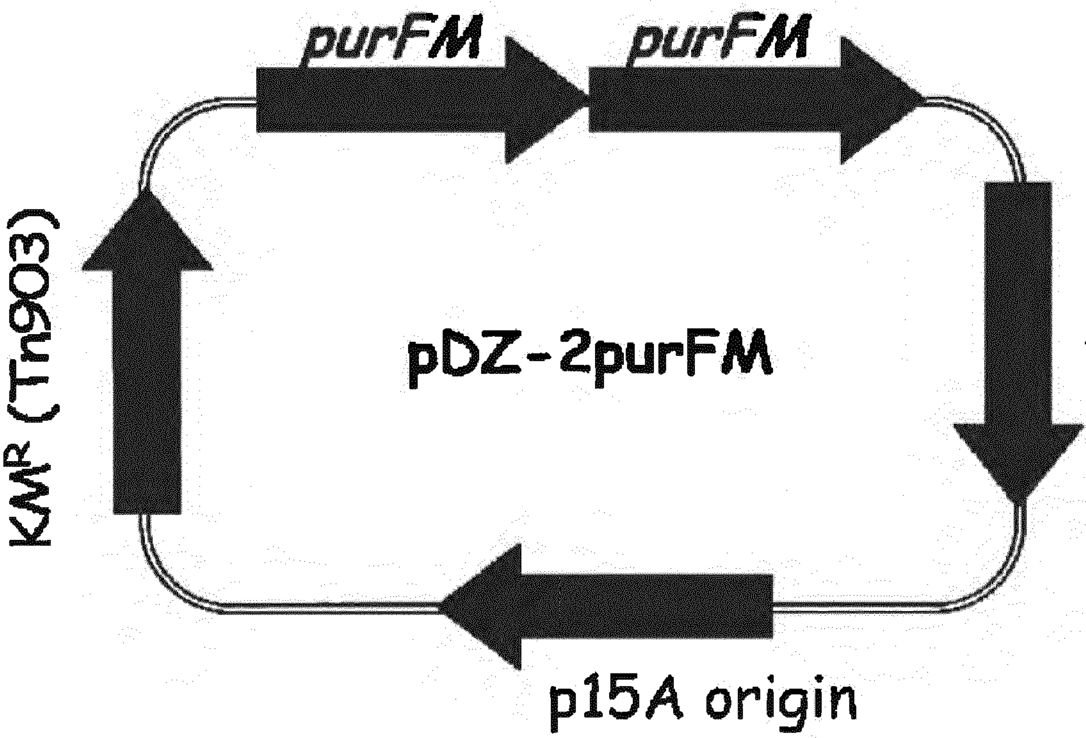
[FIG. 2]



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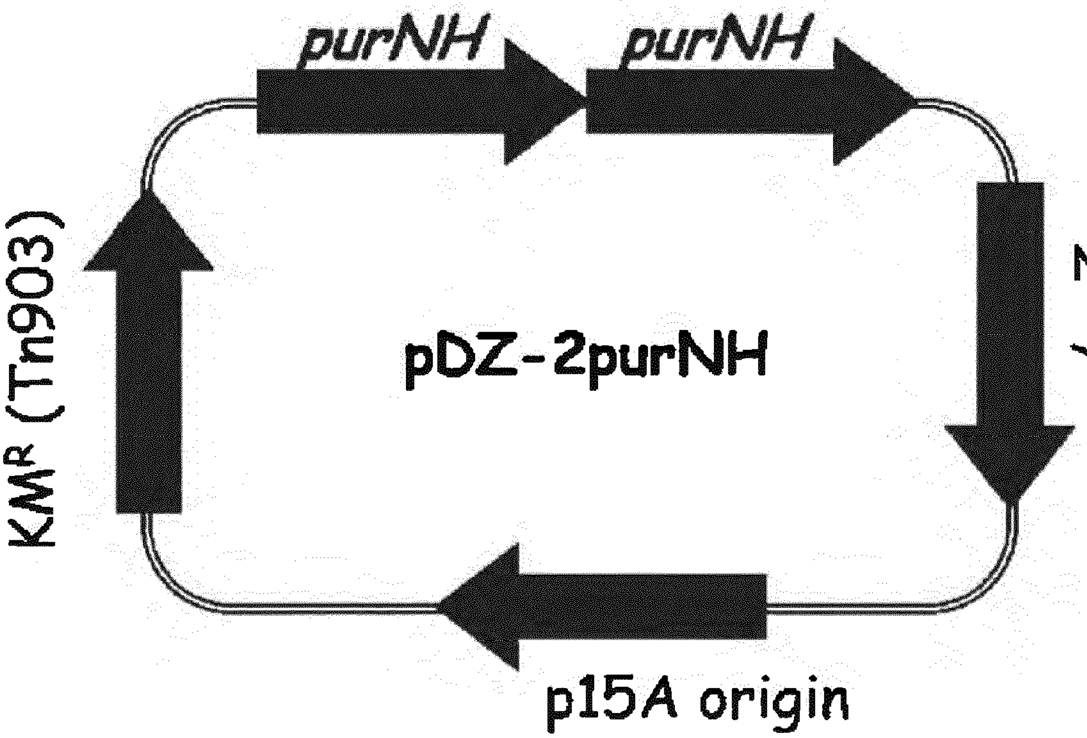

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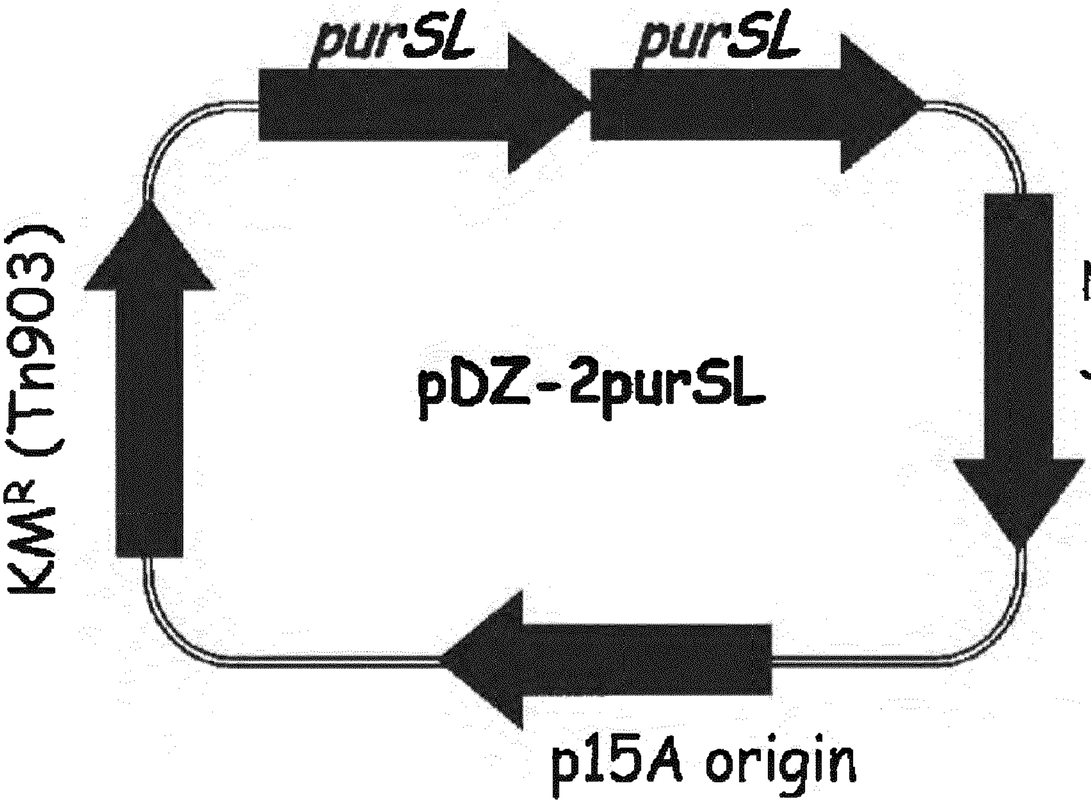

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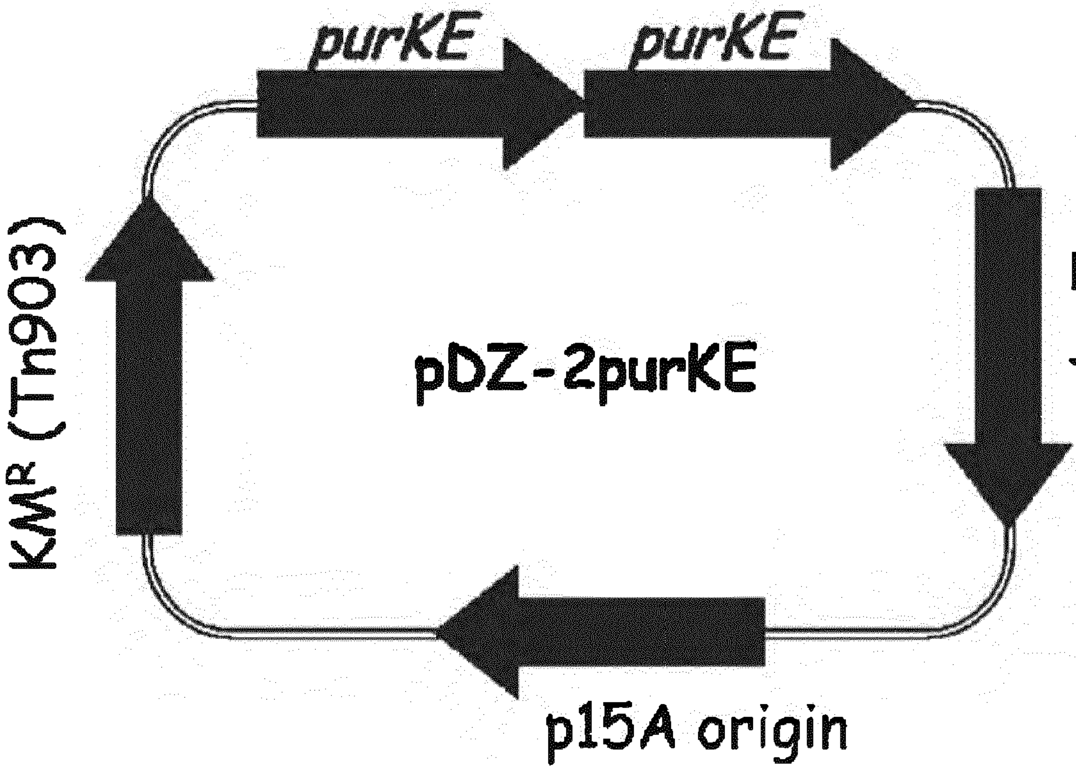

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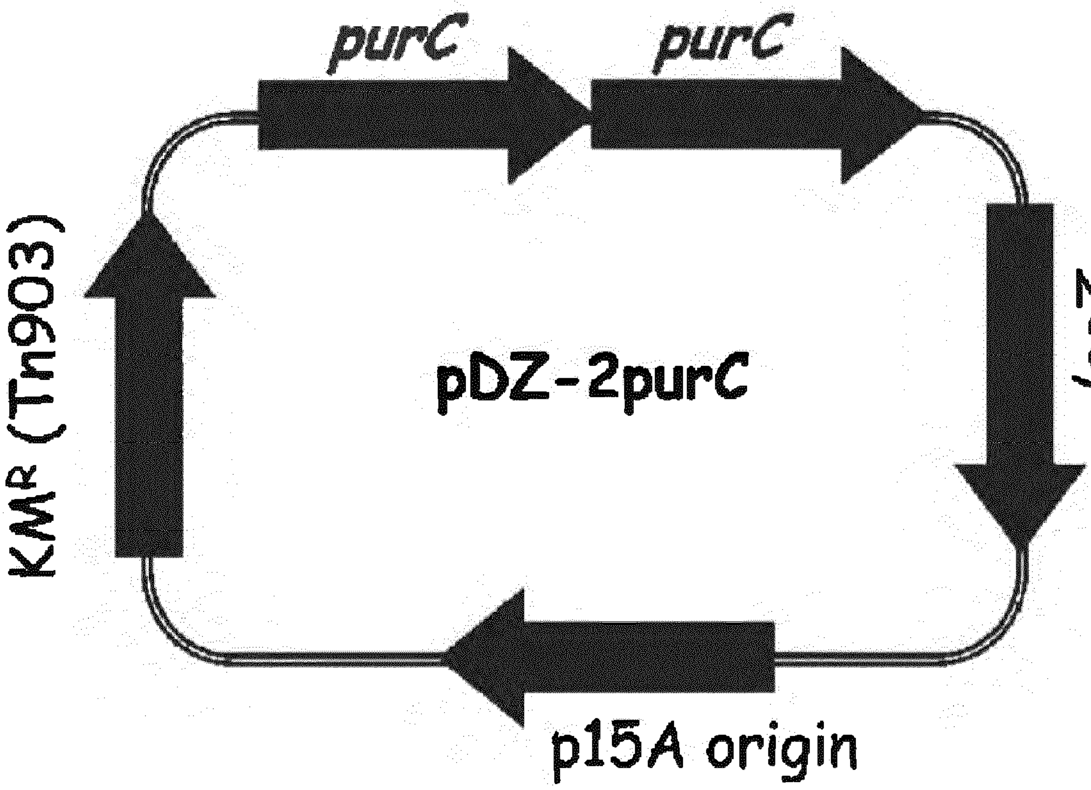

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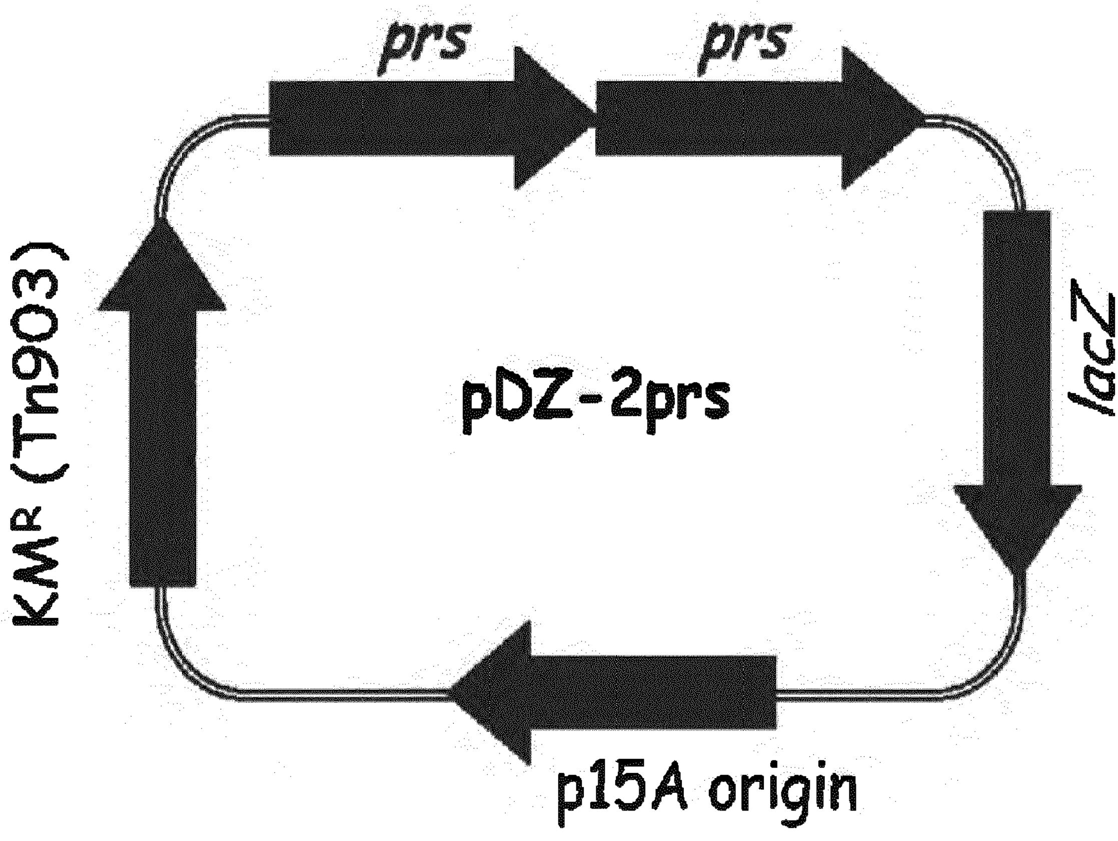

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EUROPEAN SEARCH REPORT

Application Number
EP 17 16 8260

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Y	EP 2 011 860 A1 (AJINOMOTO KK [JP]) 7 January 2009 (2009-01-07) * the whole document * -----	1-5	
Y	TOMITA K ET AL: "STIMULATION BY L PROLINE OF 5' INOSINIC ACID PRODUCTION BY MUTANTS OF CORYNEBACTERIUM-AMMONIAGENES", AGRICULTURAL AND BIOLOGICAL CHEMISTRY, JAPAN SOC. FOR BIOSCIENCE, BIOTECHNOLOGY AND AGROCHEM, TOKYO, JP, vol. 55, no. 9, 1 January 1991 (1991-01-01), pages 2221-2226, XP002234343, ISSN: 0002-1369 * the whole document * -----	1-5	
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			C12N C12P C12R

The present search report has been drawn up for all claims

1

Place of search	Date of completion of the search	Examiner
Munich	30 May 2017	Roscoe, Richard

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30-05-2017

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